PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

R. CHANEY et al.

Application No: 09/437,607

Filed: November 10, 1999

For: RECOVERING METALS FROM SOIL

Art Unit No.: 1638

Examiner: M. Ibrahim

Atty. Dkt. No.: 108172-00037

DECLARATION UNDER 37 C.F.R. §1.132

Commissioner of Patents
Washington, D.C. 20231

Sir:

I, Yin-Ming Li, of Potomac, MD, declare and state that:

I am one of the inventors in the above-identified application and I have read and understood the Office Action of June 3, 2003 and the rejections cited against the pending claims.

My comments with respect to the claimed invention are as follows:

1) Typically increasing metal availability in soil is counterproductive when the plant being used for phytoextraction does not have the metal tolerance found in natural metal-hyperaccumulator plants.

2) In the claimed invention, the response of natural-hyperaccumulator plants including *Alyssum* to soil pH is unique. Our work with numerous varieties of natural hyperaccumulator plants has yielded, to our knowledge, the first report of shoot metal increasing with increasing soil pH.

3) In the few previous reports on the effects of soil pH on metal hyperaccumulation, only the usual response of more metal uptake at lower pH has been seen. For example the Zn and Cd hyperaccumulator *Thlaspi caerulescens* (2) was reported to have decreased metal uptake at levels within the range described in the present invention compared to pH levels beneath the described range. Brown et al. (1). Moreover decreased metal uptake was reported to have been directly correlated with higher soil ph levels and conversely increased metal uptake was reported to have been directly correlated with lower soil ph levels.

4) Likewise, among non-hyperaccumulator plants, the universal response is increased shoot metal with decreasing soil pH. Zaurov et al. (2), for example, in a paper describing the effect of altering soil pH on uptake of cadmium by *Brassica juncea* grown on cadmium-contaminated soil, reported that decreasing soil pH to 5.5 (from the normal 6.5) resulted in increased cadmium concentration in plant shoots.

5) Yet, in field experiments conducted on nickel-contaminated soil in Ontario, Canada, we have grown *Alyssum* in the same plots with the agricultural crops corn, soybeans, oats, and radish. When soil pH was raised by liming, nickel concentration in *Alyssum* was raised, while that of the crop species was lowered (unpublished results).

6) Clearly, the plant species used by Raskin et al. in the U.S. Patent No. 5,785,735 (3) are not natural metal-hyperaccumulator plants as used in the claimed invention. That is, the plants of Raskin et al. do not yield increases in shoot metal with increases in soil pH, as in the claimed invention.

7) In repeated experiments we were able to demonstrate that various

Brassicaceae and *non- Brassicaceae* species were able to achieve the levels of metal hyperaccumulation described in the claimed invention when grown on soils with a pH range of 5.6 to 9.5. This effect was not limited to nickel as our experiments established that this effect extended to other metals as well. In contrast, all of the non-hyperaccumulators we tested demonstrated the exact opposite effect as their rates of metal accumulation were inhibited by the pH range described in the current invention.

8) It has been well documented that soil pH is an important factor affecting absorption of Ni and Co by crop plants. However, to our knowledge, no studies to characterize the effect of altering the pH of contaminated soils on Ni and Co accumulation by Ni/Co- hyperaccumulating *Alyssum* species have been reported. In our experiments two *Alyssum* Ni- hyperaccumulator species were used in both a greenhouse and a field experiment with farm soils collected near a historic Ni refinery in Ontario, Canada. A muck soil total Ni 1700 mg/kg while a mineral soil had 2550 mg/kg. Soil pH was adjusted using nitric acid and $CaCO_3$ to provide a range of pH before planting; the field study was conducted using similar soils and two pH levels: "as is" and calcareous by limestone addition. An unexpected result of both experiments was that Ni uptake by these *Alyssum* species was reduced at lower soil pH and increased at higher soil pH, opposite the effect on soil Ni solubility. For *A. murale* grown on Quarry muck, shoot Ni concentrations of *A. murale* were 4630 mg/kg at pH 6.32, 4600 mg/kg at pH 6.04, 3140 mg/kg at pH 5.75 and 2180 mg/kg at pH 5.4. In a subsequent field study, limestone treatment significantly increased Ni concentration in *Alyssum* shoots compared to the control (2500 to 4000 mg/kg). These results were in direct contrast

with the increased uptake of metals at lower soil pH data reported in literature for non-hyperaccumulator plants. These results indicate that pH management will be an important factor in commercial phytoextraction, and that natural Ni hyperaccumulator species achieve increased Ni accumulation at higher pH, opposite the effect of pH on Ni solubility.

9) To determine whether the observed pH effect was limited to the *Brassicaceae* family or hyperaccumulators of the *Brassicaceae* family we conducted an experiment to observe nickel uptake from serpentine soil by three hyperaccumulator species, Alyssum corsicum (*Brassicaceae* hyperaccumulator), *Alyssum murale* (*Brassicaceae* hyperaccumulator), and *Berkheya coddii* (an unrelated non-*Brassicaceae* hyperaccumulator from the *Asteraceae* family) compared with a non-nickel tolerant *Brassicaceae* species ('New Jersey Wakefield' cabbage) across a wide range of soil pH. The results of the first experiment were as follows: A. *corsicum*, *A. murale* and *B. coddii* showed significant Ni uptake at the untreated soil pH of 6.2. An equal uptake was shown at the higher pH of 6.8 and decreased uptake was shown at the acid pH (5.5) Cabbage showed signs of toxicity stress at all levels except highly calcareous. This experiment suggests that the effect of raising the pH to increase Ni uptake by hyperaccumulators is dependent on soil ion interactions. An optimum range of pH does exist where hyperaccumulators can increase the amount of Ni translocated into the shoots. This pH range would be useful for commercialization methods of phytomining so farmers may maximize Ni uptake per crop yield. This experiment also confirmed that the pH effect was not limited or related to the *Brassicaceae* family but

rather was specific to hyperaccumulators

10) A second experiment was conducted in which the hyperaccumulators, *Alyssum corsicum* (*Brassicaceae* hyperaccumulator)and *Berkheya coddii* (an unrelated non-*Brassicaceae* hyperaccumulator from the *Asteraceae* family), were compared to cabbage (the nonhyperaccumulating *Brassica oleracea*) grown in MES-HEPES buffered nutrient solutions and maintained at four pH levels (5.6, 6.2, 6.8 and 7.5) and two Ni concentrations (31.6 M and 316 M). This experiment followed an earlier pot experiment comparing a Brockman serpentine soil with two Ni-smelter contaminated soils where soil specific ion interactions played a significant role in determining the effect of soil pH on Ni hyperaccumulation. This study was designed to characterize the effect of solution pH on plant Ni uptake independent of soil ionic interactions. *A. corsicum* and *B. coddii* showed hyperaccumulation of Ni in shoots increased with solution pH. *B. oleracea* showed signs of Ni-phytotoxicity stress at all pH levels except pH 7.5 where Ni accumulation was significantly reduced. At a pH of 5.8 *A. corsicum* had a Ni concentration of 6000 parts per million; *B. coddii* had a Ni concentration of 2840 parts per million; and *B. oleracea* did not achieve hyperaccumulation but rather exhibited a Ni concentration of 720 parts per million. At a pH of 6.2 *A. corsicum* had a Ni concentration of 7710 parts per million; *B. coddii* had a Ni concentration of 4630 parts per million; and *B. oleracea* did not achieve hyperaccumulation but rather exhibited a Ni concentration of 584 parts per million. At a pH of 6.9 *A. corsicum* had a Ni concentration of 9510 parts per million; *B. coddii* had a Ni concentration of 4540

parts per million; and *B. oleracea* did not achieve hyperaccumulation but rather exhibited a Ni concentration of 384 parts per million. At a pH of 7.5 *A. corsicum* had a Ni concentration of 13800 parts per million; *B. coddii* had a Ni concentration of 6430 parts per million; and *B. oleracea* did not achieve hyperaccumulation but rather exhibited a Ni concentration of 281 parts per million. The soils where these hyperaccumulator species evolved are usually pH neutral which strongly reduces soil solution metal levels, yet they accumulate metals at levels that provide defense against pests. Hyperaccumulators may have evolved a unique mechanism to accumulate high shoot metals at increasingly alkaline pH levels despite reduction of soil metal solubility with increasing pH. These findings suggest that a hyperaccumulator plant's root membrane uptake carrier optimum is at higher pH levels than other species. This finding suggests an important component of metal hyperaccumulation allowed these species to accumulate metals at pH levels which occur naturally where the crop evolved, a pattern very different from crop plants. Similarly these findings confirm that metal hyperaccumulators which evolved in soils whose neutral pH strongly reduced available metal levels nevertheless have developed various mechanisms to accumulate metals from soils which exhibit alkaline pH levels and reduced metal solubility. Moreover it appears that acidic pH levels can inhibit the uptake mechanisms developed by hyperaccumulator species thus decreasing uptake despite increased metal solubility which typically increases metal uptake in nonhyperaccumulating species.

11) In order to determine whether the observed pH effect was limited to nickel we conducted additional experiments involving other metals. For example, we utilized

Alyssum Corsicum and *Alyssum Murale* to establish that cobalt hyperaccumulation could be achieved in soils whose pH was within the range described in the claimed invention. Thus at a pH of 6.55 *A. Murale* exhibited cobalt hyperaccumulation (1320 parts per million) as did *A. Corsicum* (1080 parts per million) while other nonhyperaccumulator species such as *Brassica Juncea* did not (25.5 parts per million).

12) The data from other experiments we conducted confirm that the rates of hyperaccumulation described in the claimed invention can be achieved by following the methods claimed. Thus, for example, Zinc hyperaccumulation rates in excess of 10,000 parts per million and Cadmium hyperaccumulation rates in excess of 100 parts per million can be achieved by utilizing some of the species (such as *Thlaspi caerulescens*) described in the claimed invention in the accordance with the methods claimed.

13) I hereby declare that all statement made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of title 18 of the United States Code and that such wilful false statements may jeopardize the validity of the application or any patent issued thereon.

Dec. 8, 2003

Date

Yin-Ming Li

References:

1. Brown, S. L.; Chaney, R. L.; Angle, J. S.; Baker, A. J. M. 1994. Phytoremediation Potential of *Thlaspi caerulescens* and *Bladder Campion* for Zinc- and cadmium-contaminated J. Environ. Qual. 23, 1151-1157.

2. Zaurov, D. E.; Perdomo, P.; Raskin, I. 1999. Optimizing soil fertility and pH to maximize cadmium removed by Indian mustard from contaminated soils. J. Plant Nutrition 22, 977-986.

3. Raskin, Ilya; Kumar, Nanda P. B. A.; Douchenkov, Slavik. 1998. Phytoremediation of metals. U.S. Patent, No. 5,785,735.